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Cellular segregation of feline leukemia provirus and viral RNA in leukocyte subsets of long-term experimentally infected cats

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Abstract

Cats exposed to feline leukemia virus (FeLV) may develop different outcomes of the infection. However, during acute infection blood proviral and viral RNA loads of cats with progressive and regressive infection are not significantly different. Thus, not the overall loads but rather those of specific leukocyte subsets may influence the infection outcome. In the present study we established the methods to determine FeLV proviral and viral RNA loads in specific leukocyte subsets. In addition, they were applied to analyze long-term persistently FeLV-infected (p27positive) and FeLV exposed but nonantigenemic (p27-negative), nonviremic cats. In the latter animals, $CD4^+$ and B lymphocytes exhibited the highest proviral loads, whereas in p27-positive cats, all leukocyte subsets showed similar high loads. In p27-positive cats, monocytes and granulocytes bore the highest viral RNA loads, whereas only one p27-negative cat was positive for viral RNA in T lymphocytes. To our knowledge, this is the first study to investigate FeLV proviral and viral RNA loads in leukocyte subsets of FeLV exposed cats. The herein described methods are important prerequisites to gain a deeper insight into the pathogenesis of FeLV infection.

Keywords

Feline leukemia virus; Quantitative real-time PCR; FACS; Leukocytes.

1. Introduction

Feline leukemia virus (FeLV) is a naturally occurring gammaretrovirus in cats. It can cause both cytoproliferative (e.g. lymphomas, myeloproliferative disorders) and cytosuppressive (e.g. immunodeficiency, anemia) diseases. Altogether, four different courses of infection have been described (Hoover and Mullins, 1991; Lutz et al., 1983b): (i) progressive infection with persistent viremia, regressive infection with (ii) transient or (iii) undetectable viremia, and (iv) localized FeLV-infection in tissues, such as spleen, lymph nodes, small intestine and mammary glands without concurrent viremia (Hayes et al., 1989; Pacitti et al., 1986). Latent nonproductive infection characterized by the absence of viremia and persistence of the virus in the bone marrow has been described in cats that ostensibly recovered from a regressive infection (Madewell and Jarrett, 1983; Rojko et al., 1982). Categorization of cats into these groups is typically accomplished by immunofluorescence assays (Hardy et al., 1973b; Hoover and Mullins, 1991; Jarrett et al., 1982) as well as by FeLV p27 antigen ELISA from blood plasma (Lutz et al., 1983a); antigenemia is a marker of infection and in most but not all cats a parameter for viremia (Jarrett et al., 1982). Recently, Torres and co-workers re-examined the FeLV-host relationship and suggested the four putative categories progressive, latent, regressive and abortive infection based on the results of real-time Taqman® PCR and p27 ELISA (Torres et al., 2005). Moreover, most recently, the spectrum of host response categories was also refined by investigating plasma viral RNA loads in FeLV exposed cats (Hofmann-Lehmann et al., In press; Hofmann-Lehmann et al., 2006).

In spite of the common use of vaccines in veterinary practices, FeLV infections are still highly prevalent. A few years ago, 7% of the Swiss pet cat population was p27- as well as provirus-positive and exhibited high proviral loads as assessed by quantitative real-time PCR (Hofmann-Lehmann et al., 2001); 10% of the cats tested provirus-positive, but p27-negative. The latter animals had significantly lower proviral loads than the p27-positive cats (Hofmann-

Lehmann et al., 2001). The p27-negative but FeLV-positive status, characterized by low proviral load, was also demonstrated in vaccinated cats, where integration of the provirus took place in spite of protection from persistent viremia (Cattori et al., 2006).

The susceptibility of cats to FeLV is age-dependent: 100% of the cats experimentally infected as newborn kittens and 85% inoculated at 2 weeks to 2 months of age became persistently viremic, but only 15% of the cats that were inoculated when they were 4 months or older (Hoover et al., 1976). Furthermore, the status of the immune system plays an important role in FeLV infection: immunosuppressive agents and chemical carcinogens have been shown to increase the susceptibility of adult cats to FeLV (Hoover et al., 1981; Rojko et al., 1982; Schaller et al., 1979). Virus neutralizing antibodies (VNA) as well as FeLV-specific cytotoxic T lymphocytes (CTL) play an important role in the protection of cats against FeLV. VNA can protect against persistent viremia (Jarrett et al., 1977), and cats with regressive viremia developed significantly higher antibody titers than persistently infected animals (Hofmann-Lehmann et al., 2001). But in the majority of cases, VNA appeared at the time when cats that had shown transient viremia recovered (Flynn et al., 2000; Hofmann-Lehmann et al., 2006). In contrast, virus-specific CTL were present as early as one week postexposure, and cats vaccinated with a DNA vaccine were protected from infection without developing VNA (Flynn et al., 2000). In addition and in agreement with another study (Poulet et al., 2003), 8 out of 10 cats vaccinated with a non-adjuvant canarypox-vectored live vaccine containing FeLV-A *env*, *gag* and part of *pol* (Eurifel®, Merial, Lyon, France) were protected from viremia without developing antibodies prior to experimental FeLV infection (Hofmann-Lehmann et al., 2006). In persistently infected cats, neither VNA nor high levels of FeLV specific CTL were detected (Flynn et al., 2002; Flynn et al., 2000; Hoover and Mullins, 1991)

Cats which developed a persistent viremia showed higher proviral and viral loads than transiently viremic cats, but not prior to week 2 and week 1 postexposure, respectively

(Hofmann-Lehmann et al., 2001; Hofmann-Lehmann et al., 2006). It thus seems that cats which are ultimately able to overcome the infection initially fight similar high proviral and viral loads in the peripheral blood like cats that become persistently infected. This led us to assume that not the overall viral loads in the peripheral blood, but rather different loads in specific leukocyte subsets influence the outcome of the infection. So far, only FeLV antigen loads could be measured in specific leukocyte subsets; proviral and viral RNA loads e.g. in antigen-negative cats could not be determined because of lack of adequate methods.

It was thus the goal of the present study to establish a method to sort feline leukocytes by flow cytometry and quantify the proviral and viral RNA loads in sorted cell subsets by means of real-time PCR and reverse transcriptase-PCR (RT-PCR). In addition, the method was applied to analyze long-term infected p27-positive and p27-negative SPF cats.

2. Materials and Methods

2.1. Animals and virus inoculation

Eleven three-year-old specific pathogen free, barrier-maintained cats (Charles River Laboratories, Lyon, France) were used in this study (Table 1). They were housed in groups in large rooms under optimal ethological and hygienic conditions. The cats were challenged intraperitoneally with FeLV-A/Glasgow-1 (family *Retroviridae*, subfamily *Orthoretrovirinae*, genus *Gammaretrovirus*, species *Feline leukemia virus*, strain *A/Glasgow-1* (Hofmann-Lehmann et al., 2006)) 2.5 years prior to the present investigation. Five of the cats have become persistently infected: they tested provirus-positive in real-time PCR (Tandon et al., 2005), were viremic as determined by virus isolation (Jarrett and Ganiere, 1996) and antigenemic as determined by double sandwich ELISA (Lutz et al., 1983a). Six cats were provirus-positive, but nonviremic and nonantigenemic. The presence of infectious FeLV was determined *in vitro* by inoculation of heparinized plasma samples onto QN10S cells (Jarrett and Ganiere, 1996). Plasma VNA were measured by focus reduction of FeLV-A/Glasgow-1

in QN10S cells (Jarrett and Ganiere, 1996). For each blood sample, the total and differential white and the red blood cell counts were determined in EDTA-anticoagulated blood using an electronic cell counter (Cell-Dyn 3500, Abbott, Baar, Switzerland).

2.2. Preparation and staining of leukocytes and cell sorting

Prior to staining procedures and fluorescent activating cell sorting (FACS), peripheral blood mononuclear cells (PBMC) were purified from 6 ml of EDTA-anticoagulated blood by Percoll gradient centrifugation. Briefly, blood was centrifuged at 170 × g for 10 min at room temperature (RT); the plasma was removed and replaced by the same volume of Hank's balanced salt solution (HBSS, Invitrogen, Basel, Switzerland). Then 4 ml of 43% Percoll (Amersham Biosciences, Uppsala, Sweden) was added to a 14 ml centrifuge tube (TPP, Trasadingen, Switzerland), and underlayered with 4 ml of 62.5% Percoll. The blood/HBSS solution was layered onto the Percoll gradient and the tubes were centrifuged at RT for 10 min at 514 × g followed by 20 min at 914 × g. The mononuclear cell layer was harvested, washed once in HBSS and resuspended in 100 µl phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) and 2 mM EDTA.

Because the number of monocytes in the peripheral blood is particularly low, they were further enriched prior to FACS analysis using the MiniMACS Starting Kit® (Miltenyi Biotech, Bergisch Gladbach, Germany). Briefly, Percoll purified PBMC were labeled with an antibody against CD14 (TüK4, Dako Cytomation, Zug, Switzerland) recognizing monocytes (Bienzle et al., 2003; Willett et al., 2003), incubated for 20 min at RT and washed once with PBS containing 0.5% BSA and 2 mM EDTA. Cells were then stained with rat anti-mouse immunglobulin G (IgG) MicroBeads (Miltenyi Biotech) and run through a MS Column® (Miltenyi Biotech). Magnetic separation led to the collection of two fractions: $CD14^+$ enriched cells and the remainder of the PBMC. The $CD14^+$ enriched cells were labeled in the dark at RT for 20 min with a dichlorotriazinylaminofluorescein (DTAF)-conjugated

secondary antibody ($F_{(ab)}$ goat anti-mouse IgG [H&L], Jackson ImmunoResearch Laboratories, West Grove, PA, USA).

The rest of the PBMC were washed, resuspended in PBS containing 1% BSA and 0.1% NaN_3 and underwent staining as follows. The cell suspension was divided into aliquots and put in 5 ml Falcon polystyrene tubes (catalog number 352058, BD Biosciences, Allschwill, Switzerland). Cells were then incubated for 20 min at RT in the dark with one of the following antibodies: an unconjugated mouse anti-feline CD4 antibody (Fel7, Southern Biotech, Birmingham, Alabama, USA) (Ackley et al., 1990), an unconjugated mouse anti-feline CD8 antibody (FT2, Southern Biotech) (Klotz and Cooper, 1986), a fluoresceinisothiocyanate (FITC)-conjugated mouse anti-feline CD5 antibody (f43, Southern Biotech) recognizing T lymphocytes (Dean et al., 1991), and a Peridinin chlorophyll-a Protein (PerCP)-conjugated rat anti-mouse CD45R/B220 antibody (RA3-6B2, BD Bioscience) recognizing B lymphocytes (Brown et al., 2002; Willett et al., 2003). CD5 was used because in the domestic cat, CD5 expression is restricted to T cells (Ackley and Cooper, 1992; Brown et al., 2002). Prior to labeling of the B lymphocytes, purified rat IgG (Southern Biotech) was added to block all Fc receptors and prevent nonspecific binding. The $CD4^+$ and $CD8^+$ cells were labeled in the dark at RT for 20 min with a DTAF-conjugated goat anti-mouse secondary antibody.

For granulocyte preparation, blood was collected in a 2 ml Vacutainer (BD Biosciences) prefilled with 11 glass beads (Bender&Hobein, Zurich, Switzerland) and 0.4 ml of a 6% dextran-solution in 0.9% NaCl. After collection, the blood was gently rotated for 5 min in order to remove the platelets. Defibrinated blood (100 µl) was put into a 5 ml Falcon polystyrene tube and incubated with a purified goat IgG (Southern Biotech) for 10 min at RT to block the Fc receptors. Granulocytes were then labeled for 20 min at RT with unconjugated mouse anti-feline granulocyte antibodies (CL35A, VMRD Inc., Pullman, WA, USA) followed by a DTAF-conjugated goat anti-mouse secondary antibody for an additional 20 min

at RT in the dark. Finally hypotonic formic acid (550 µl) and, after 10 sec, 250 µl of a salt solution (57 mM Na_2CO_3, 248 mM NaCl and 220 mM Na_2SO_4 in 1L ddH_2O) were added to the tube while vortexing to lyse the red blood cells.

In each experiment, negative controls were included as follows. For indirect staining, cells were incubated with only the secondary antibody. As a control for direct staining, cells were incubated with an isotype-matched non-binding antibody: for $CD5^+$ lymphocytes, FITC-conjugated mouse IgG1 isotype control (clone 15H6, Southern Biotech), and for B lymphocytes, PerCP-conjugated rat IgG_{2a} monoclonal immunoglobulin isotype control (clone R35-95, BD Bioscience).

Stained cells were stored at 4°C and filtrated through a 50 µm filter (LabForce, Nunningen, Switzerland) immediately prior to sorting on a BD FACS Aria, and analyzed using the BD FACS Diva software™. On the basis of the forward versus side scatter, 10,000 cells were gated to set the fluorescence cutoff; this was set so that less than 2% of the negative events were included in the positive analysis region. Cells were sorted into PBS and frozen at -80°C until DNA extraction.

2.3. DNA extraction and FeLV/GAPDH real-time PCR

DNA was extracted from sorted cells and from 100 µl EDTA-anticoagulated blood using the QIAmp® DNA Micro Kit (Qiagen, Hombrechtikon, Switzerland). DNA was eluted in 50 µl elution buffer and stored at -80°C until use. FeLV provirus was quantified by real-time PCR as described (Tandon et al., 2005). In addition, feline glyceraldehyde-3-phosphate dehydrogenase (GAPDH), of which one copy of a pseudogene is present in the genomic DNA of feline cells (Molia et al., 2004), was quantified in each sample by real-time PCR (Leutenegger et al., 1999). FeLV proviral copies per cell were obtained by dividing the FeLV copy number by the GAPDH copy number (Cattori et al., 2006).

2.4. RNA extraction and FeLV/GAPDH real-time RT-PCR

From sorted cells, viral RNA was extracted from the first flow-through (250 µl) of the respective DNA extraction using the MagnaPure LC mRNA Isolation Kit (Roche, Basle, Switzerland). The same kit was used to extract RNA from 100 µl EDTA-anticoagulated blood. In addition, viral RNA was extracted from 280 µl of plasma with the QIAamp® Viral RNA Mini Kit (Qiagen). RNA samples were stored at -80°C until use. FeLV viral RNA loads of the leukocyte subsets as well as of the EDTA-anticoagulated blood and plasma were determined as published (Tandon et al., 2005). Viral RNA loads in leukocyte subsets and whole blood were normalized to GAPDH mRNA. Plasma viral RNA loads were calculated as copies per ml of plasma.

2.5. Statistics

Data were compiled and analyzed with Prism software (GraphPad, San Diego, CA). For the comparison of FeLV proviral and viral RNA loads among different leukocyte subsets, the Kruskal-Wallis 1-way ANOVA by Ranks (pKW) and the Dunn's post test (pD) were used. Loads and cell numbers of p27-negative and p27-positive cats were compared using the Mann-Whitney U Test (pMWU). Differences were considered significant if $p < 0.05$.

3. Results

3.1. Characteristics of the cats

Infectious FeLV was found in the p27-positive, but not in the p27-negative cats (Table 1). Plasma viral RNA was detected in the p27-positive and in one of the p27-negative animals (Table 1). Within the p27-negative cats, five out of five animals tested developed VNA; in contrast, none of the p27-positive cats had VNA. No significant difference was found in total leukocyte, lymphocyte, granulocyte, and monocyte counts between p27-positive and p27-negative cats (Table 2). When the numbers of cells that had been sorted from a constant input

blood volume were compared, long-term p27-positive cats had slightly lower $CD4^+$ and $CD5^+$ T-cell counts than p27-negative cats (both pMWU = 0.0823). No difference or tendency was found when the numbers of the other sorted cell subsets were compared.

3.2. Proviral loads in specific leukocyte subsets

Cats that were p27-positive had significantly higher blood proviral loads (median load = 3.74×10^6 copies/10^6 cells) than p27-negative cats (median load = 4.68×10^1 copies/10^6 cells; pMWU = 0.0043; Fig. 1). In addition, a significant difference was also found when the proviral loads were calculated per ml of blood: p27-positive cats had higher loads (median load = $1.30x10^7$ copies/ml) than p27-negative cats (median load = $1.53x10^2$ copies/ml; pMWU = 0.0043). Proviral loads of leukocyte subsets were significantly different within the p27-negative cats, in that the $CD4^+$ cells had significantly higher loads (median load = 1.15×10^2 copies/10^6 cells) than the granulocytes (median load = 0 copies/10^6 cells; pKW = 0.0112; $pD < 0.05$; (Fig.1). A significant difference was also found between the proviral loads of lymphocytes (median load = 5.98×10^1 copies/10^6 cells) and the remaining white blood cells (monocytes and granulocytes, median load = 0 copies/10^6 cells; pMWU = 0.0260). In the group of p27-positive cats, no significant difference was found among proviral loads of different leukocyte subsets (pKW = 0.0622; Fig.1).

3.3. Viral RNA loads in specific leukocyte subsets

A significant difference was found between blood viral RNA loads of p27-positive (median load = 6.49 copies/GAPDH copy) and p27-negative cats (median load = 0 copies/GAPDH copy; pMWU = 0.0043; Fig. 2) as well as in plasma viral RNA loads between p27-positive (median load = $1.23x10^8$ copies/ml) and p27-negative cats (median load = 0 copies/ml; pMWU = 0.0043; Table 2).

In the group of the p27-positive cats, all leukocyte subsets were highly positive for FeLV RNA (Fig. 2). The viral RNA loads of the leukocyte subsets within this group were

significantly different and $CD4^+$ cells had significantly lower loads (median load = 3.9×10^{-1} copies/GAPDH copy) than monocytes (median load = 4.83 copies/GAPDH copy; pKW = 0.0031; pD < 0.01). Viral RNA loads of monocytes and granulocytes (median load = 3.46 copies/GAPDH copy) were significantly higher than the loads in lymphocytes (median load = 1.36 copies/GAPDH copy; pMWU = 0.0079). Viral RNA was only found in T lymphocytes and in the plasma of one of the six p27-negative cats (Fig.2).

4. Discussion

To our knowledge, this is the first study to quantify FeLV DNA and RNA loads in different feline leukocyte subsets of FeLV exposed cats. Our results demonstrate that FeLV proviral and viral RNA loads are distinctively distributed among the leukocyte subsets of long-term infected p27-negative and p27-positive cats, respectively.

In accordance with earlier reports (Hofmann-Lehmann et al., 2001; Hofmann-Lehmann et al., 2006; Torres et al., 2005), long-term p27-positive cats had higher overall FeLV proviral and viral RNA loads than p27-negative cats. The cats under investigation had been infected for 2.5 years with FeLV-A/Glasgow-1; while p27-positive and p27-negative cats had still similar total peripheral white blood counts, the p27-positive cats had somewhat reduced $CD4^+$ T-cell numbers. Although it may be assumed that these results were not very precise because the method was not primarily designed to measure absolute cell numbers, they are in agreement with an earlier investigation with the same virus where $CD4^+$ T-cell counts were found reduced in long-term persistently FeLV-infected cats (Hofmann-Lehmann et al., 1997). The described method for the determination of FeLV proviral and viral RNA loads in individual leukocyte subsets combines flow cytometric cell sorting with sensitive real-time TaqMan PCR and the loads are normalized against GAPDH copy numbers as a measure for the cell number (pseudogene) and the transcription level of a house keeping gene, respectively. Thus,

the reduced $CD4^+$ T-cell counts of persistently infected cats did not influenced our investigations of absolute FeLV loads in individual cell subsets.

Rojko and coworkers have described six sequential phases of FeLV infection (Rojko et al., 1979). In cats with regressive viremia, the control of the virus seems to take place at phase 3 (viral replication in lymphoid germinal cells in lymphoid tissues throughout the body) and phase 4 (viral replication in bone marrow neutrophil and platelet precursor cells and crypt epithelium). These cats exhibit only a first cell-associated viremia in lymphocytes and monocytes, but not a second viremia of bone marrow origin, which is characterized by the presence of FeLV group-specific antigen (GSA, *gag* gene coded protein p27) in neutrophils and platelets (Hardy et al., 1973a; Hoover et al., 1977). In agreement with that, our data indicate that in regressively infected cats, no second viremia and therefore no infection of granulocytes took place. However, our study exceeds these data in that beside the lack of viral protein expression in granulocytes of p27-negative cats (Rojko et al., 1979), also no provirus was detectable in this leukocyte subset. In addition, we demonstrate that in cats with undetectable antigenemia, certain cell subsets, in particular $CD4^+$ and B lymphocytes may still be provirus positive even 2.5 years after FeLV exposure.

Most former studies which aimed to identify viral target cells used immunofluorescence to detect FeLV GSA in blood leukocytes, lymph nodes, bone marrow and epithelial tissue (Hardy et al., 1973a; Hoover et al., 1987; Hoover et al., 1977; Rojko et al., 1978; Rojko et al., 1979). In the present study, we used new sensitive methods to find productively infected white blood cells. Cats with productive infection showed higher viral RNA loads in monocytes and granulocytes when compared to those in lymphocytes. Remarkably, in persistently infected cats lymphocytes and in particular $CD4^+$ T cells were infected to a lesser degree than other cell subsets although $CD4^+$ cells were found to be decreased in long-term FeLV infection (see above and (Hofmann-Lehmann et al., 1997)). It may thus be speculated that either not a direct effect of FeLV infection leads to the destruction of $CD4^+$ lymphocytes

or only a subset of these cells reacts particularly sensitive to FeLV infection. The finding that particularly monocytes and granulocytes but to a lesser degree lymphocytes are infected is in agreement with other studies: The most frequent and most intense specific fluorescence was found in the cytoplasm of neutrophils, monocytes and in platelets in blood smears of viremic cats, either naturally or experimentally infected with FeLV (Hardy et al., 1973b; Hoover et al., 1977). Quackenbush and coworkers have shown that in cats chronically infected with FeLV-FAIDS 61E or acutely infected with FeLV-FAIDS 61E/C, almost all of the feline myeloid antigen positive monocytes and granulocytes expresses FeLV p27 (85 – 93%), but only a fraction (20 – 60%) of $CD4^+$, $CD8^+$ and IgG^+ lymphocytes (Quackenbush et al., 1996). Because of the good agreement with published data, we assume that our method primarily detects productively infected cells, although we cannot completely rule out that some of the viral RNA sequences detected in granulocytes by real-time TaqMan PCR originated from viral particles that had been take up via phagocytosis.

In the group of the p27-negative cats, only one cat was positive for plasma viral RNA and for cell-associated viral RNA in T lymphocytes. The presence of viral RNA might be indicative for replication of the virus in this particular cat, although the animal tested negative in p27 ELISA and virus isolation. There might be several explanations for this discrepancy. Firstly, the sensitivity of p27 ELISA and virus isolation might be lower than that of the RT-PCR. Therefore p27 ELISA and virus isolation could give false negative results for cats with a very low-level infection. Secondly, the cat might have had a localized infection and virus particles, which were defective and not replication competent were released into the peripheral blood. These particles might not be detectable by p27 ELISA or virus isolation. In contrast, the presence of free RNA in the plasma seems less likely due to low stability.

Enriched peripheral blood polymorphonuclear leukocytes of FeLV-infected cats show a significantly lower chemiluminescence and a decreased phagocytic and oxidative burst activity than those of noninfected control cats (Hoffmann-Jagielska et al., 2005; Lafrado and

Olsen, 1986; Lewis et al., 1986). This was also the case for neutrophils of FeLV-exposed cats, which were nonviremic (Lafrado and Olsen, 1986). The latter observation could be explained by the finding that purified viral protein p15 acted as a immunosuppressive agent *in vitro* (Hebebrand et al., 1979; Mathes et al., 1979) and a synthetic analogue of p15E was demonstrated to suppress the respiratory burst of human monocytes (Harrell et al., 1986). In addition, data presented by Lafrado and coworkers (Lafrado et al., 1987) indicate that ultraviolet light-inactivated FeLV and the purified protein p15E impairs the function of feline neutrophils. Thus, not the productive infection of granulocytes, but rather the presence of viral proteins or parts of viral proteins seems to affect the function of neutrophils in FeLV-exposed cats. In agreement with this, it may be assumed that also in the nonviremic cats of the present study, the granulocyte function might have been decreased although the cells were FeLV provirus-negative.

In p27-positive cats, the FeLV proviral loads in most of the cells exceeded 1 copy per cell, whereas in the majority of p27-negative cats, some leukocyte subsets, such as $CD8^+$ lymphocytes, monocytes and granulocytes, were found provirus-negative. This was even the case when up to 1.7×10^5 granulocytes were sorted and a very sensitive real-time PCR assay (Tandon et al., 2005) (detection limit: 1 copy/5 µl reaction) was used. The inability to detect provirus in these cells could be explained by the fact that the proviral loads were nevertheless below the detection limit of our assay. However, provirus was detectable in other leukocyte subsets despite of very low cell numbers (minimum of 5×10^4 cells). In addition, our data are in agreement with the findings of Rojko and coworkers who have shown that FeLV GSA cannot be detected in neutrophils of transiently infected cats, but in those of persistently infected cats (Rojko et al., 1979).

In conclusion, this study was performed to establish the necessary methods to assess proviral and viral RNA loads in feline cell subsets and to investigate the magnitude of the FeLV infection in different cell subsets of persistently infected and nonviremic FeLV exposed cats.

Using sensitive molecular methods, the proviral and viral RNA loads of specific leukocyte subsets were analyzed and significant differences between and within leukocyte subsets of long-term infected, p27-positive and p27-negative cats were found. The particular distribution of FeLV provirus and viral RNA during long-term infection may originate from differences in the very early phase of the infection, which seems crucial for the clinical outcome. To further address the hypothesis that the initial loads in specific leukocyte subsets influence the course of infection, the proviral and viral RNA loads should be investigated during the very first few weeks after infection.

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Table 1

Virological and immunological characteristics of p27-positive and p27-negative cats

Cat ID	**11**	**12**	**20**	**49**	**57**	**69**	**19**	**32**	**10**	**43**	**38**
p27-ELISA (%)		0	0	0	0	0	0	83	71	60	109	75
Virus isolation		neg[a]	neg	neg	neg	ND[b]	neg	pos[c]	pos	pos	pos	pos
Titer of VNA		1:32	1:32	1:32	1:32	ND	1:32	0	0	0	0	0
Plasma viral RNA[d]		2.58×10^4	0	0	0	0	0	1.1×10^8	1.2×10^7	8.9×10^7	4.0×10^8	4.1×10^8

[a]neg = negative, [b]ND = not done, [c]pos = positive, [d]FeLV RNA copies/ml plasma

Table 2

Hematological characteristics of p27-positive and p27-negative cats

Cat ID	**11**	**12**	**20**	**49**	**57**	**69**	**19**	**32**	**10**	**43**	**38**	
Leukocytes (10^3/µl)	3.22	3.93	8.45	7.99	5.60	9.05	4.27	3.53	4.09	5.09	4.78	
Lymphocytes (10^3/µl)		1.02	1.52	5.48	3.90	1.43	4.02	2.52	1.19	0.98	1.02	1.13
Monocytes (10^3/µl)		0.09	0.17	0.09	0.34	2.00	0.45	0.10	0.25	0.02	0.11	0.22
Granulocytes (10^3/µl)		2.11	2.24	2.88	3.74	4.03	4.59	1.65	2.09	3.09	3.96	3.44
Erythrocytes (10^6/µl)	7.89	6.80	7.91	9.08	6.96	8.82	7.73	8.00	7.58	7.45	8.14	
Hemoglobin (g/dl)		11.9	11.3	13.0	12.5	10.9	12.6	11.3	12.2	12.2	11.2	12.7
PCV (%)[a]	33.6	32.1	36.4	37.9	32.0	37.7	32.8	36.0	35.2	32.2	36.2	

[a] PCV = packed cell volume

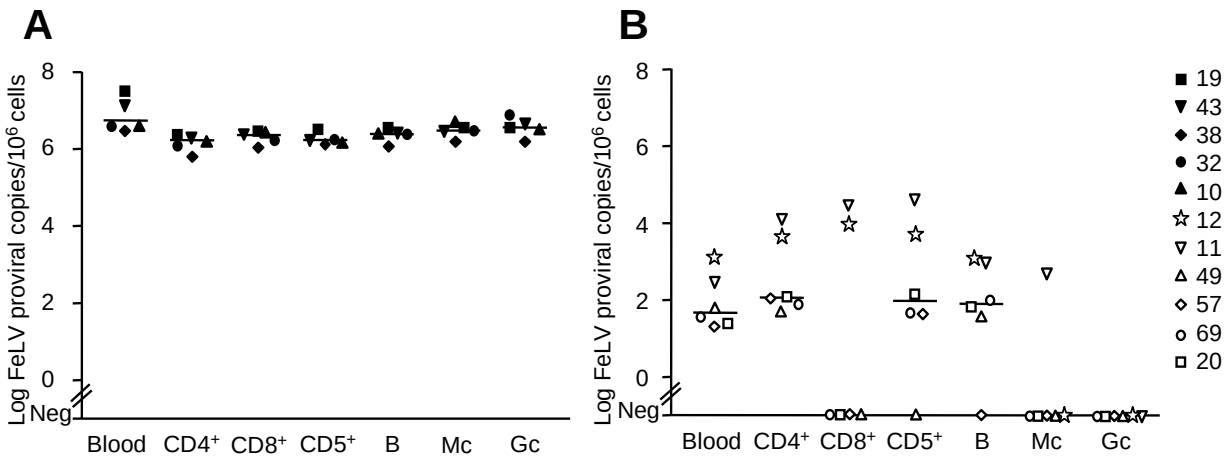

Fig. 1: FeLV proviral loads in EDTA-anticoagulated whole blood (blood) and specific leukocyte subsets of p27-positive (A) and p27-negative (B) cats.
CD4+, CD4+ lymphocytes; CD8+, CD8+ lymphocytes; CD5+, CD5+ lymphocytes; B, B lymphocytes; Mc, monocytes; Gc, granulocytes. The figure legend depicts the symbols corresponding to the different cats as listed in Tables 1 and 2.

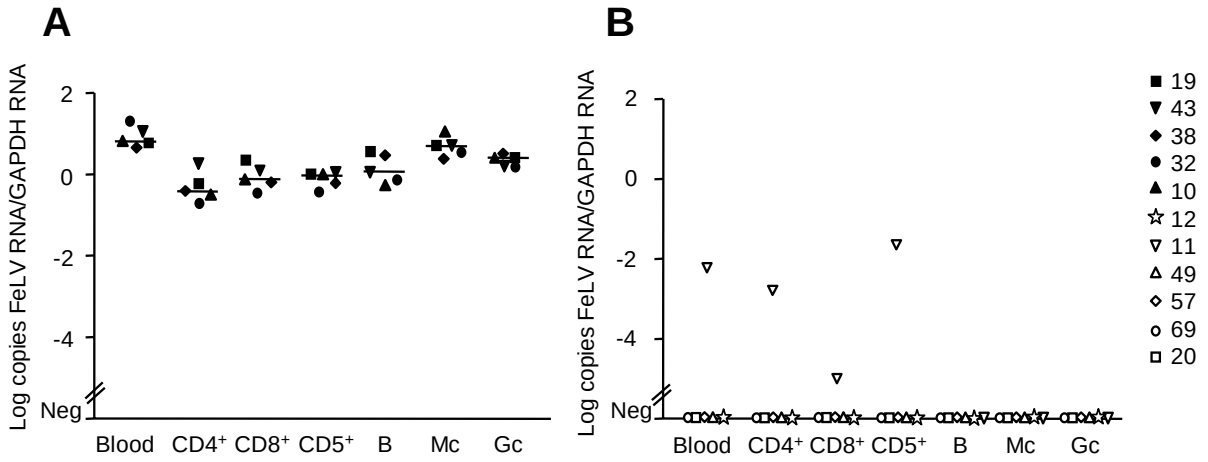

Fig. 2: FeLV viral RNA loads in EDTA-anticoagulated whole blood (blood) and specific leukocyte subsets of p27-positive (A) and p27-negative (B) cats.
CD4+, CD4+ lymphocytes; CD8+, CD8+ lymphocytes; CD5+, CD5+ lymphocytes; B, B lymphocytes; Mc, monocytes; Gc, granulocytes. The figure legend depicts the symbols corresponding to the different cats as listed in Tables 1 and 2.

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